Biomarkers for diagnosing Alzheimer's disease

Field of the invention

5 The invention is in the field of diagnostics. More specifically, the invention is in the field of assessing the state of Alzheimer's disease in subjects by detection of Alzheimer's disease-specific marker polypeptides.

Background of the invention

10

Alzheimer's disease

Alzheimer's disease is an increasingly prevalent form of neurodegeneration that accounts for approximately 50-60% of the overall cases of dementia among people

15 over 65 years of age. Pathologically, Alzheimer's disease neurodegeneration is characterised by prominent atrophy of corticolimbic structures with neuronal death and loss of neuronal synapses, neurofibrillary tangle (NFT) formation, and the formation of senile plaques containing deposits of amyloid $\beta1$-42 (Aβ42) aggregates in the brain [Francis PT 1999]. The duration of the progressive cognitive decline is

20 approximately 7 years from the occurrence of first signs until death. It is assumed that the clinical phase is preceded by a 15-30 years preclinical period of continuous deposition of amyloid plaques and neurofibrillary tangles. Age of onset and progression of the disease are largely determined by causative gene mutations and by genetic susceptibility factors. Several environmental risk factors may add to the

25 individual genetic risk factors. Genetic factors known to be involved in the familial form of Alzheimer's disease with early onset of the disease are: mutations in presenilin 1 (PS1), presenilin 2 (PS2), and amyloid precursor protein (APP) genes, and the presence of the apolipoprotein E4 allele. However, the majority (95%) of Alzheimer's disease cases is sporadic and heterogeneous.

30

Currently, clinical diagnosis of Alzheimer's disease can only be established at later stages of the disease, when cognitive performance is significantly decreased and paralleled by structural alterations of the brain. The clinical diagnostic work up requires a careful medical history; physical and neurological examination; blood, urine and cerebrospinal fluid (CSF) examinations to exclude metabolic and medical disease states that might masquerade Alzheimer's disease; detailed psychometric examinations to assess mental status and cognitive performance, and imaging techniques such as computed tomographic scan or magnetic resonance imaging of the brain. Diagnostic evaluations at expert centres reach an accuracy of about 80-85%.

Due to the fact that these tests are expensive and time consuming, and are particularly inconvenient to patients, there is an increasing need for easy-accessible specific diagnostic biomolecule markers, which can be measured in body fluids, such as CSF, blood or urine, and which have a high positive predictive value for diagnosis of Alzheimer's disease, or would help to distinguish Alzheimer's disease from other forms of dementia. Furthermore, reliable markers sensitive to disease progression may constitute surrogate parameters, a major prerequisite for the evaluation and development of new causal oriented and disease modifying therapeutic strategies in Alzheimer's disease.

Since CSF directly surrounds the brain, changes in its protein composition may most accurately reflect pathologic conditions that are associated with specific alterations of the protein expression patterns. Over the last decade, a number of biological abnormalities have been reported in the cerebrospinal fluid (CSF) of Alzheimer's disease patients, in particular altered levels of the Aß1-42 fragment of the amyloid precursor protein, and altered levels of the hyperphosphorylated tau protein. The sensitivity and specificity of these markers, however, is low or only modest [The Ronald and Nancy Reagan Research Institue of the Alzheimer's Association and the National Institute on Aging Working Group, 1998, Robles A 1998, Teunissen CE et al., 2002].

Hence, there is a need for novel biomarkers with sufficient sensitivity and specificity for (i) detecting Alzheimer's disease as early as possible, and (ii) to allow disease differentiation from other types of dementia or neurodegenerative diseases, and (iii) monitoring therapeutic efficacy as surrogate parameter, e.g. in clinical drug

5   development, and to initiate pharmacotherapy as early as possible and postpone loss of memory and disease progression.

Protein Chip Technology

10   A Protein chip technology called Surface Enhanced Laser Desorption/Ionisation time of flight mass spectrometry (SELDI-TOF MS) has recently been developed to facilitate protein profiling of complex biological mixtures [Davies HA 2000, Fung ET 2001, Merchant M 2000].

15   Protein chip mass spectrometry has already been used by several groups to detect potentially novel biomarkers of prostate and bladder [Adam BL 2001] or breast cancer [Wulfkuhle JD 2001] in serum, seminal plasma, nipple fluid, urine or cell extracts. For a review on biomarker search using SELDI-TOF MS, see [Issaq HJ 2002].

20

Cystatin C

Initially described in 1961 in cerebrospinal fluid (CSF), cystatin C (γ trace or post-γ globulin, Acc. No. P01034) is a small cystein proteinase inhibitor present in all

25   human body fluids at physiologically relevant concentrations. The physiological role of cystatin C is likely to regulate extracellular cysteine protease activity, which results from microbial invasion or release of lysosomal proteinases from dying or diseased cells. Cystatin C colocalises with ß-amyloid (Aß) within the arteriolar walls in Alzheimer's disease brains and cerebral amyloid angiopathy [Levy E 2001]. There

30   are two common haplotypes of the CST3 gene coding for cystatin C (A and B) that differ from each other at three sites: two single base pair changes in the promoter

region and one in the signal peptide domain that causes an amino acid substitution (alanine to threonine). Recently, case control studies found associations of CST3 with increased risk for late onset Alzheimer's disease [Crawford FC 2000, Finckh U 2000, Beyer K 2001].

Hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I), also called hereditary cystatin C amyloid angiopathy (HCCAA), is an autosomal dominant form of cerebral amyloid angiopathy (CAA). The amyloid deposited in the brain vessel's walls is composed mainly of a variant of cystatin C characterised by the presence of the Leu68-Gln substitution [Cohen 1983, Ghiso 1986]. This pathology is also coupled to a decreased concentration of this major cystein proteinase inhibitor in cerebrospinal fluid and leads to its amyloid deposition in the brain [Grubb AO 1984].

Leung-Tack *et al* have also purified two N-terminal truncated isoforms of cystatin C in urine from one patient who had received renal transplant. According to their data, (des1-4) cystatin C has an inhibiting effect on two functions of human peripheral mononuclear cells (PMN): O_2^- release and phagocytosis, which may be due to the N-terminal sequence 'KPPR'. Their data support a potentially important role for cystatin C as a possible immunomodulator during inflammation. Accumulating evidence indicates that increased free radical mediated damage to cellular function contributes to the ageing process and age-related neurodegenerative disorders. Oxidative stress may play a role in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS). Although free-radical damage to neurons may not be the primary event initiating these diseases, it appears that free-radical damage is involved in the pathogenetic cascade of these disorders.

Beta-2-microglobulin

Beta-2-microglobulin (Acc. No. P01884) constitutes the small constant component of the class I major histocompatibility complex (CMH) and its presence in biological

fluids represents the balance between membrane protein turnover and elimination. Since this peptide seems to be increased in some diseases characterised by an elevation of the immune response, its quantification in body fluids has become a useful index of immunological state in vivo [Hoekman et al 1985]. The function of this protein is unclear, but it seems to be implicated in diseases, which involve glial cell destruction [Ernerudh et al 1987].

The technical problem which is solved by the present invention is the provision of improved methods for diagnosing Alzheimer's disease and/or monitoring the progression of Alzheimer's disease in a subject.

Neurosecretary Protein (VGF)

VGF (human VGF, Acc.-No.: O15240) is a secretory peptide precursor that is expressed and processed by neuronal cells [Canu et al. 1997]. In situ hybridization studies in the adult rat central nervous system have revealed that the VGF mRNA is widely distributed throughout the brain with prominent expression in the hippocampus, entorhinal cortex, and neocortex. Furthermore, it has been shown that VGF transcription and secretion is selectively upregulated by neurotrophins like NGF and BDNF, and by depolarization *in vitro*. Increased BDNF expression can be observed in dentate gyrus and CA3 regions of the hippocampus, which are tissues that appear to die early in Alzheimer Disease pathogenesis.

Description of the invention

The invention is based on the surprising finding that specific polypeptides are differentially expressed in subjects having Alzheimer's disease when compared to a healthy control group. These differentially expressed polypeptides can be, e.g., detected in samples of cerebrospinal fluid of the subject in which Alzheimer's disease is to be diagnosed. The individual polypeptides of the invention can be detected and/or quantified alone or in combination with other polypeptides of the invention.

- 6 -

The polypeptide markers of the invention are defined by their respective molecular weight. Five markers identified by the SAX2 method as described in the Examples show the following molecular masses:

        Marker 1 (M1): 4824 ± 20 Da;

        Marker 2 (M2): 7691 ± 20 Da;

        Marker 3 (M3): 11787 ± 20 Da;

        Marker 4 (M4): 11988 ± 20 Da;

        Marker 5 (M5): 13416 ± 20 Da.

Table one shows the observed molecular weight of polypeptide markers M1 to M5 as determined by SELDI-TOF MS, the amino acid sequences of observed fragments of polypeptide markers M1 to M5, and the protein from which the polypeptide markers M1 to M5 originate.

Table 1:

Marker	SELDI observed MW	Amino Acid Sequence	Protein Name
M1	4823.5 Da ± 1.7	VGEEDEEAAEAEAEAEEAER	VGF4.8
M2	7691.4 Da ± 4.9	XXAD(L/I)AGHG(Q/K)EV(L/I)(L/I)R · HGTVV(L/I)TA(L/I)GG(L/I)(L/I)K	human myoglobin new variant
M3 M4	11786.9 Da ± 7.6 11988.4 Da ± 5.9	VNHVTLSQPK VEHSDLSFSK IEKVEHSDLSFSK SNFLNCYVSGFHPSDIEVDLLK	human beta-2-microglobulin
M5	13416.4 Da ± 9.4	ASNDMYHSR ALDFAVGEYNK RALDFAVGEYNK LVGGPMDASVEEEGVR QIVAGVNYFLDVELGR LVGGPMDASVEEEGVRR KQIVAGVNYFLDVELGR TQPNLDNCPFHDQPPHLK TQPNLDNCPFHDQPPHLKR SSPGKPPRLVGGPMDASVEEEGVR	human Cystatin C

The differentially expressed polypeptides of the invention can be, e.g., detected in samples of cerebrospinal fluid of the subject in which Alzheimer's disease is to be diagnosed. In addition, depending on the specific embodiment, the source of samples to measure the abundance of the polypeptide markers of the invention, can also be

5   blood, serum, or urine, but is not limited to these body compartments.

As shown in Figure 1, compared to a negative diagnosis (healthy controls), M1 is under-expressed in the CSF of Alzheimer's disease patients $(p < 0.05)$, while the markers M2 to M5 are over-expressed in CSF of Alzheimer's disease patients $(p <

10   0.05)$.

An altered level of one or several polypeptides of the invention, compared to the level of polypeptides of the invention in healthy control subjects, will allow assessing the state of and/or monitoring the progression of Alzheimer's disease in a subject,

15   will allow monitoring the effectiveness of Alzheimer's disease treatment, and will be useful information for drug development. Furthermore, these biomolecule markers are useful for differentiating Alzheimer's disease from other forms of dementia and neurodegenerative disorders.

20   Preferred subjects in which Alzheimer's disease is to be diagnosed or monitored are human subjects. However, diagnosis of Alzheimer's disease according to the invention is also possible with other mammals. If necessary, orthologues of the peptide markers of the invention can be used.

25   The invention also relates to the use of mass spectrometry (MS) for detecting Alzheimer's disease in human subjects and for assessing the progression of Alzheimer's disease in human subjects by detecting and/or quantifying the amount of specific polypeptides in samples drawn from the subject's body fluids. In a preferred embodiment of the invention, the sample is drawn from the subject's cerebrospinal

30   fluid (CSF).

Detection and/or quantification of the polypeptides of the invention is preferably achieved by quantifying the signal which is detected by MS at specific molecular mass to charge ratios (M/z) which correspond to the M/z ratios of the polypeptides of the invention. Preferably, M/z ratios close to the one of the polypeptides of the

5   invention are also measured.

Detection and/or quantification of the polypeptides of the invention can also be achieved by using an immunoassay using specific antibodies raised against the specified marker(s) or polypeptide fragments thereof. Antibodies can be prepared by

10   using the purified marker(s) or fragments thereof, or using synthetic or re-combinantly expressed polypeptide(s) consisting of the specific amino acid sequence of the marker(s) using any suitable method known in the art [Coligan 1991]. Such techniques include, but are not limited to, antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or appropriate vectors,

15   as well as preparation of polyclonal and monoclonal antibodies by immunising rabbits or mice [Huse 1989, Ward 1989]. After the antibody is provided, a marker can be detected and/or quantified using any of a number of standard immunological binding assays [US Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168]. Useful assays include, but are not limited to, for example, an enzyme immune assay (EIA)

20   such as enzyme-linked immunosorbent assay (ELISA), a radioimmune assay (RIA), a Western blot assay, or a slot or dot blot assay. For a review of the general immunoassays see [Coligan 1991]. Generally, a sample obtained from a subject can be contacted with the antibody that specifically binds the marker. A powerful technique to capture the specified marker(s) from a complex body fluid sample is to

25   use the antibody fixed to solid supports, such as glass or plastic, e.g. microtiter plate, a stick, a bead, or microbead. Alternatively, marker(s) can also be captured from the body fluid sample by the specific antibody immobilised to a probe substrate or a ProteinChip™ array, as described for the SELDI-based immunoassay [Xiao 2001]. After incubating the sample with antibodies, the non-bound material is washed under

30   specified conditions and the antibody-marker complex formed can be detected, using appropriate detection reagents. In an embodiment using the SELDI ProteinChip™

- 9 -

array technique the marker(s) selectively enriched by the immobilised antibody can be detected and quantified by matrix-assisted laser desorption ionisation mass spectrometry.

5   The invention specifically relates to

1.   a method of assessing the state of Alzheimer's disease in a subject comprising detection of at least one polypeptide comprised in a group of polypeptides consisting of

10

i)   a polypeptide having a molecular mass of 4824 ± 20 Da,

ii)   a polypeptide having a molecular mass of 7691 ± 20 Da,

iii)   a polypeptide having a molecular mass of 11787 ± 20 Da,

iv)   a polypeptide having a molecular mass of 11988 ± 20 Da, and

15   v)   a polypeptide having a molecular mass of 13416 ± 20 Da.

The invention further relates to a method of assessing the state of Alzheimer's disease in a subject comprising detection of at least one polypeptide comprised in a group of polypeptides having, respectively, molecular masses of 4824 ± 20

20   Da, of 7691 ± 20 Da, of 11787 ± 20 Da, of 11988 ± 20 Da, of 13416 ± 20 Da, of 4769 ± 20 Da, of 6958 ± 20 Da, of 6991 ± 20 Da, of 13412 ± 20 Da, of 13787 ± 20 Da, of 17276 ± 20 Da, of 40437 ± 20 Da, of 6895 ± 20 Da, of 6928 ± 20 Da, of 7691 ± 20 Da, of 7769 ± 20 Da, of 7934 ± 20 Da, of 5082 ± 20 Da, of 6267± 20 Da, of 6518 ± 20 Da, of 7274 ± 20 Da, and of 8209 ± 20 Da.

25

Whereas detection of one such polypeptide is in most cases sufficient to reliably diagnose Alzheimer's disease, detection of two or more polypeptides of the invention can increase the sensitivity and robustness of the method. Preferably, 1, 2, 3, 4, 5, 10, and, most preferred, all of said polypeptides will be

30   detected from the same sample. The detection can also be carried out simultaneously with the detection of other polypeptides which are preferably

also differentially expressed in subjects having Alzheimer's disease as compared to healthy subjects. "Assessing the state of Alzheimer's disease" shall be understood as diagnosing the presence of Alzheimer's disease in a subject or a patient, as assessing the progression of the disease in a subject or a patient, and/or as assessing the proneness of a subject to develop Alzheimer's disease.

2.   The invention further relates to the method of point 1 in which 2, or 3, or 4, or 5 polypeptides of said group of peptides are detected. The invention further relates to a method of point 1 in which 2, or 3, or 4, or 5, or 10 or all polypeptides of said group of peptides are detected.

3.   The invention further relates to a method of assessing the state of Alzheimer's disease in a subject comprising detection of at least one polypeptide comprising the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and/or SEQ ID NO:16. The invention further relates to a method of assessing the state of Alzheimer's disease in a subject comprising detection of at least one polypeptide comprising the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, and/or SEQ ID NO:17. Whereas detection of one such polypeptide is in most cases sufficient to reliably diagnose Alzheimer's disease, detection of two or more polypeptides of the invention can increase the sensitivity and robustness of the method. Preferably, 1, 2, 3, 4, 5, 10, or all of said polypeptides will be detected from the same sample. The detection can also be carried out simultaneously with the detection of other polypeptides which are preferably also differentially expressed in subjects having Alzheimer's disease as compared to healthy subjects.

4.   The invention further relates to a method of assessing the state of Alzheimer's
disease in a subject comprising detection of at least one polypeptide comprised
in a group of polypeptides consisting of

5   i)   human cystatin C,

ii)   human beta-2-microglobulin,

iii)   human myoglobin (new variant),

iv)   a fragment of at least 5, 8, 10 , or 20 amino acids of human cystatin C,

v)   a fragment of at least 5, 8, 10 , or 20 amino acids of human beta-2-

10   microglobulin, and

vi)   a fragment of at least 5, 8, 10 , or 20 amino acids of human myoglobin
(new variant).

The invention further relates to a method of assessing the state of Alzheimer's

15   disease in a subject comprising detection of at least one polypeptide comprised
in a group of polypeptides consisting of

i)   human cystatin C,

ii)   human beta-2-microglobulin,

20   iii)   human myoglobin (new variant),

iv)   human neurosecretory protein VGF,

v)   a fragment of at least 5, 8, 10 , or 20 amino acids of human cystatin C,

vi)   a fragment of at least 5, 8, 10 , or 20 amino acids of human beta-2-
microglobulin,

25   vii)   a fragment of at least 5, 8, 10 , or 20 amino acids of human myoglobin
(new variant), and

viii) a fragment of at least 5, 8, 10 , or 20 amino acids of neurosecretory
protein VGF.

30   Whereas detection of one such polypeptide is in most cases sufficient to
reliably diagnose Alzheimer's disease, detection of two or more of said

- 12 -

polypeptides can increase the sensitivity and robustness of the method. Preferably, 1, 2, 3, 4, 5, or 6 of said polypeptides will be detected from the same sample. More preferably, 1, 2, 3, 4, 5, 6 or all of said polypeptides will be detected from the same sample. The detection can also be carried out simultaneously with the detection of other polypeptides which are preferably also differentially expressed in subjects having Alzheimer's disease as compared to healthy subjects.

5.   The invention further relates to a method of investigating the progression of Alzheimer's disease in a subject characterised in that a method of any of points 1 to 4 is performed with at least two distinct samples drawn from the same subject. For this purpose, samples drawn from a subject at different points in time will be analysed. Changes in the amount of the respective polypeptide(s) will allow to draw conclusions on the progression of Alzheimer's disease in the subject.

6.   The invention further relates to a method of any of points 1 to 5, wherein detection of said polypeptide(s) is by SELDI-TOF MS. Other suitable mass spectrometric methods and other methods of detection can alternatively be used. More specifically, the invention relates to a method of any of points 1 to 5, wherein detection of said polypeptide(s) is by SELDI-TOF MS in which the hydrophobic H50, the WCX2, or the IMAC surface is used as a support upon ionisation. Different supports for ionisation yield different sensitivity for specific proteins of interest.

7.   The invention further relates to a method of any of points 1 to 5, wherein specific antibodies or antibodies recognising said polypeptide(s) are used for detection of said polypeptide(s).

8.   The invention further relates to a method of any of points 1 to 7, wherein detection is in a sample comprising CSF of said patient. A sample drawn from

a subject can be processed immediately after it has been taken, or it can first be frozen and be analysed later. Samples may also consist of or contain other body fluids such as blood, serum, plasma, urine, seminal plasma, nipple fluid, or cell extracts.

9.   The invention further relates to a kit comprising a polypeptide having a molecular mass of 4824 ± 20 Da, a polypeptide having a molecular mass of 7691 ± 20 Da, a polypeptide having a molecular mass of 11787 ± 20 Da, a polypeptide having a molecular mass of 11988 ± 20 Da, and/or a polypeptide having a molecular mass of 13416 ± 20 Da. The invention further relates to a kit comprising a polypeptide having a molecular mass of 4824 ± 20 Da, a polypeptide having a molecular mass of 7691 ± 20 Da, a polypeptide having a molecular mass of 11787 ± 20 Da, a polypeptide having a molecular mass of 11988 ± 20 Da, and a polypeptide having a molecular mass of 13416 ± 20 Da. The invention further relates to a kit comprising polypeptides having a molecular mass of 4824 ±± 20 Da, of 7691 ± 20 Da, of 11787 ± 20 Da, of 11988 ± 20 Da, of 13416 ± 20 Da, of 4769 ± 20 Da, of 6958 ± 20 Da, of 6991 ± 20 Da, of 13412 ± 20 Da, of 13787 ± 20 Da, of 17276 ± 20 Da, of 40437 ± 20 Da, of 6895 ± 20 Da, of 6928 ± 20 Da, of 7691 ± 20 Da, of 7769 ± 20 Da, of 7934 ± 20 Da, of 5082 ± 20 Da, of 6267± 20 Da, of 6518 ± 20 Da, of 7274 ± 20 Da, and/or of 8209 ± 20 Da.Such a kit can be applied for various purposes, e.g., for use as a standard in one of the above mentioned methods. Said kit can comprise 2, 5, 10, or all of the above polypeptides.

10.   The invention further relates to a kit comprising a fragment of at least 5 amino acids of human cystatin C, a fragment of at least 5 amino acids of human beta-2-microglobulin, and a fragment of at least 5 amino acids of human myoglobin. This kit can be applied for various purposes, e.g., for use as a standard in one of the above mentioned methods. The invention further relates to a kit comprising a fragment of at least 5, 10 or 20 amino acids of human cystatin C, a fragment of at least 5, 10 or 20 amino acids of human beta-2-microglobulin, a fragment

of at least 5, 10 or 20 amino acids of human myoglobin, and a fragment of at least 5, 10 or 20 amino acids of neurosecretory protein VGF. These kits can be applied for various purposes, e.g., for use as a standard in one of the above mentioned methods.

5

Brief description of the figures

Figure 1:

10   Average intensities of the five marker peptides of Table 1, which are differentially expressed in the diseased group when compared to the control groups.

Examples

The invention is further described by one or several of the following examples. These examples are not to be understood as restricting the scope of the invention to the examples by any means.

Example 1: Patients evaluation and CSF sampling

Diagnosis of Alzheimer's disease in human subjects was made according to criteria of the National Institute of Neurologic and Communicative Disorders and Stroke–Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA). The Alzheimer's disease group consisted of 9 patients aged 75±7 years, six men and three women. The group of healthy control subjects consisted of 10 individuals aged 78 ± 14 years, two men and eight women with no history, symptoms or signs of psychiatric or neurological disease.

Informed consent was given by each patient and the patients' caregivers before the investigation. The study was approved by the local ethics committee. After lumbar puncture, CSF samples were frozen on dry ice immediately upon withdrawal at the bedside in 0.5 ml aliquots and stored at -80°C until analyses.

Example 2: ProteinChip SELDI analysis of CSF on SAX2 chip

SAX 2 Proteinchip array (Ciphergen Biosystems, Fremont, CA, USA) were equilibrated for 5 min with 5µl of binding buffer (100mM Na Acetate pH=4.0). The buffer was carefully removed with an handkerchief and 2.5µl of binding buffer was added to the wells. Crude CSF samples (2.5µl) were added to the wells and incubated for 20 min at room temperature in a humidity chamber on a rocking platform. CSF was removed and the wells were individually washed with 10µl of binding buffer for 5 min. The arrays were then placed in a 15ml conical Eppendorf and washed twice with the binding buffer for 5 min. Finally, the chip was rinsed twice with distilled

water. Excess of H_2O was removed and while the surface was still moist, two additions per well of 0.5µl of sinapinic acid (SPA) (2mg/ml) in 50% (vol/vol) acetonitrile and 0.5% (vol/vol) trifluoroacetic acid was performed and dried. The arrays were then read in a ProteinChip reader system, PBS II series (Ciphergen Biosystems). The laser beam was focused on the sample *in vacuo*. This caused the proteins absorbed to the matrix to become ionised and, simultaneously to be desorbed from the Proteinchip array surface. The ionised proteins were detected and their molecular masses were determined according to their time-of-flight (TOF). TOF mass spectra, collected in the positive ion mode were generated using an average of 65 laser shots throughout the spot at a laser power set slightly above threshold (10-15 % higher than the threshold) High mass to acquire was set at 40kDa, optimised from 1 to 15kDa. Spectra were collected and analysed using the Ciphergen Proteinchip (version 3.0) software. External calibration of the reader was performed using the "all-in-1" peptide molecular weight standards (Ciphergen biosystems, Inc.) diluted in the SPA matrix (1:1, vol/vol) and directly applied onto a well. Protein profile comparison was performed after normalisation on total ion current of all the spectra included in the same experiment. The reproducibility was tested by analysing different aliquots of the same CSF sample on 4 different wells of the same proteinchip array (intraassay intrachip reproducibility), on two different chips (intraassay interchip reproducibility) processed in parallel, and reproduced in an other experiment (interassay reproducibility).

Analysis of CSF samples from 9 patients diagnosed with Alzheimer's disease relative to 10 controls revealed that 5 peaks were significantly differentially expressed between the two groups ($p < 0.05$). The approximate average SELDI mass associated with the five differentially expressed proteins was 4.82kDa, 7.7kDa, 11.8kDa and 12.0kDa and 13.4kDa ($p < 0.05$) (see Table 1, Fig. 1).

Example 3: Strong Anionic exchange chromatography (SAX) purification

In order to identify the proteins corresponding to these peaks, a fractionation of crude CSF on a SAX spin column was performed. The eluted fractions were analysed by
5   SELDI-TOF MS.

SAX spin column, lot number SAX2-001116-01, (Ciphergen Biosystems, Fremont, CA, USA) was rehydrated overnight at 4°C in the equilibration buffer (20mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 5mM NaCl, pH 9.0).
10   The column was warmed up at room temperature and air bubbles were removed. The equilibration buffer was let flow through column matrix by gravity. Equilibration buffer (0.5ml) was added to the column and passed through the resin twice. Two ml of control CSF was diluted in the equilibration buffer (1:1, vol/vol). Protein sample was loaded to the column by fraction of 0.8ml and allowed to run through the column
15   by gravity until no drops came out of the column. The column was then centrifuged at 150 x g for 1 min. The resin was then washed with an equivalent volume of equilibration buffer. This step was repeated several times in order to load the whole sample onto the resin. Elution of the bound proteins was performed by decreasing the pH. Elution buffer A consisted of 20mM Tris-HCl, 5mM NaCl pH 8.0; elution buffer
20   B = 20mM sodium phosphate pH 7.0; elution buffer C = 20mM sodium phosphate pH 6.0; elution buffer D = 20mM sodium phosphate and citrate pH 5.0; elution buffer E = 20mM sodium phosphate and citrate pH 4.0; elution buffer F = 20mM sodium phosphate and citrate pH 3.4; elution buffer G = 30% acetonitrile in elution buffer F. Elution was performed by applying 2 x 75 µl of the elution buffer and
25   centrifugation at 150 x g for 1min. Each collected fraction (150 µl) was concentrated on a speed-vac to a volume of 10µl. Protein profiles were analysed on SELDI-TOF MS using SAX 2 Proteinchip arrays. The chip was equilibrated with a binding buffer consisting of 20mM Tris-HCl, 5mM NaCl, pH=9.0. An aliquot of 0.5 µl of each concentrated fraction was applied directly onto 2.5µl of binding buffer per spot and
30   processed as previously described. The rest of the fractions were loaded onto a Tris tricine gel as described below.

The differentially expressed peak of 13.4kDa was eluted with buffer A (20mM Tris-HCl 5mM NaCl pH 8.0) and B (20mM sodium phosphate pH 7.0). The differentially expressed peaks of 11.8kDa and 12.0kDa were found in the fraction eluted with buffer C (20mM sodium phosphate pH 6.0) and D (20mM sodium phosphate and citrate pH 5.0). The cluster of 7.7kDa was eluted with buffer D (20mM sodium phosphate and citrate pH 5.0) and E (20mM sodium phosphate and citrate pH 4.0).

Each eluted fraction was loaded on a 16.5% Tris Tricine sodium dodecyl sulfate polyacrylamide gel and electrophoresed (SDS PAGE). After coloration with coomassie blue, the bands seen on the gel confirmed the results obtained by SELDI analysis. The band corresponding to the cluster of 7.7kDa, 11.8kDa and 12.0kDa were cut out. Proteins were extracted as described in Example 6 and identified by Q-TOF. The 7.7kDa peak MS analysis did not match with any known human protein, however, may indicate to a new variant or homologue of myoglobin. Peptide sequences were the following

XXAD(L/I)AGHG(Q/K)EV(L/I)(L/I)R and
HGTVV(L/I)TA(L/I)GG(L/I)(L/I)K.

The MS analysis of the 11.8kDa and 12.0kDa peaks identified beta-2-microglobulin for both of them.

Since the cluster of 13.4kDa could not be seen on the Tris Tricine gel, a Tris glycine SDS-PAGE electrophoresis was performed on crude CSF samples. The band corresponding to the beta-2-microglobulin could be easily found on this stained gel. We concluded that the protein migrating just above the beta-2-microglobulin could correspond to the next abundant protein seen on the SELDI profile, namely the 13.4kDa peak. The band was excised from the gel and digested by trypsin before MALDI analysis. The peptide mass fingerprint analysis allowed to identify the Cystatin C. The sequence coverage provided by the analysis was 60%.

Example 4: Monodimensional electrophoresis / Tris Glycine gels

Twenty μl of CSF were mixed with 10 μl of denaturing Laemmli buffer [Laemmli
1970]. The samples were heated to 95°C for 5 min, and loaded on a 15% T (T = total
acrylamide concentration) SDS-polyacrylamide gel according to the method of
Laemmli. Gels were stained in a solution containing Coomassie Brilliant Blue R-250
(0.1% w/v) and methanol (50% v/v) for 30 min. Destaining was done in a solution
containing methanol (40% v/v) and acetic acid (10% v/v).

Example 5: Monodimensional electrophoresis : Tris Tricine Gels

Tris tricine SDS-PAGE electrophoresis was performed according to Schagger and
von Jagow [1987] using precast 16.5 % T gels (Biorad, Hercules, CA). The anode
buffer consisted of 0.2M Tris-HCl, pH 8.9 and the cathode buffer consisted of 0.1M
Tris-HCl, 0.1M Tricine, 0.1% SDS, pH 8.25. Samples were diluted in 10μl of 50mM
Tris-HCl, 4% (w/v) SDS, 12% (w/v) sucrose, 5% (v/v) ß-mercaptoethanol, and trace
of bromophenol blue, pH 6.8. After denaturation at 95°C for 5min, samples were
loaded onto the gel. Gels were run at 80V for 3 hours. After electrophoresis, gels
were fixed in 40% methanol, 10% acetic acid for 30min. Gels were then stained with
Colloidal blue coomassie G250 overnight and destained in 30% methanol. Bands to
be identified were immediately cut, placed in an eppendorf and kept at 4°C until
further analysis. The apparent molecular masses were determined by running
polypeptide molecular weight (MW) standards: Triosephophate isomerase MW
26,625; Myoglobin MW 16,950; α-lactalbumin MW 14,437; Aprotinin MW 6,512;
Insulin b chain, oxidised MW 3,496 and Bacitracin MW 1,423 (Biorad).

Example 6: Protein digestion and peptide extraction [Bienvenu 1999]

Fragments of gels containing proteins of interest were cut out for digestion of the
proteins with trypsin using previous published procedures [Shevchenko 1996,

- 20 -

Hellman 1994, Rosenfeld 1992] and modified as described below. The piece of gel was first destained with 100 μl of 50 mM ammonium bicarbonate, 30% (v/v) acetonitrile during 15 min at room temperature. Destaining solution was removed and replaced by 25 μl of 10 mM DL-dithiothreitol (DTT) in 50 mM ammonium bicarbonate and incubated 35 min at 56°C. DTT solution was then replaced by 25 μl of 55 mM iodoacetamide in 50 mM ammonium bicarbonate and incubated during 45 min at room temperature in the dark. Gel pieces were washed for 10 min with 100 μl of 50 mM ammonium bicarbonate and for 10 min with 100 μl of 50 mM ammonium bicarbonate and 30% (v/v) acetonitrile.

Gel pieces were then dried for 30 min in a Hetovac vacuum centrifuge (HETO, Allerod, Denmark). Dried pieces of gel were rehydrated for 45 min at 4°C in 5-20 μl of a solution of 50 mM ammonium bicarbonate containing trypsin at 6.25 ng/μl. After an over-night incubation at 37°C, gel pieces were dried under high vacuum centrifuge before being rehydrated by the addition of 20 μl of distilled water and finally dried again in a speed-vac for 30 min. Extraction of the peptides was performed with 20 μl of 0.1% (v/v) trifluoroacetic acid (TFA) for 20 min at room temperature with occasional shaking. The TFA solution containing the peptides was transferred to a polypropylene tube. A second elution was performed with 20 μl of 0.1% (v/v) TFA in 50% (v/v) acetonitrile for 20 min at room temperature with occasional shaking. The second TFA solution was pooled with the first one. The volume of the pooled extracts was reduced to 1-2 μl by evaporation under vacuum. Control extractions (blanks) were performed using pieces of gels devoid of proteins.

Example 7: Protein identification by peptide mass fingerprinting analysis

1.5 μl of sample was placed on a MALDI 100-well target plate. Same volumes of matrix (10 mg/ml α-Cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile, 0.1% (v/v) TFA) were added to the previously loaded digest. Samples were dried as quickly as possible using a vacuum container. Mass measurement from liquid solution were conducted with a MALDI-TOF mass spectrometer Voyager™ Elite

and Super STR (PerSeptive Biosystems, Framingham MA, USA) equipped with a 337 nm nitrogen laser. The analyser was used in the reflectron mode at an accelerating voltage of 20 kV, a delayed extraction parameter of 100-140 ns and a low mass gate of 850 Da. Laser power was set slightly above threshold (10-15 % higher than the threshold) for molecular ion production. Spectra were obtained by summation of 10 to 256 consecutive laser shots. Masses of the 60 highest peaks were extracted from the spectra and used for protein identification using the SmartIdent peptide mass fingerprint tool [Gras 1999]. The research was conducted against SWISS-PROT and TrEMBL databases. The query was made for the human, the minimum number of matched masses was 4, the maximal tolerance for masses was 50 ppm after an internal calibration using autolysis product of trypsin, at most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carboxymethylation with iodoacetamide of cysteines and artefactual oxidation of methionines.

Example 8: Protein identification by peptide fragmentation analysis

Prior to nanoLC (LC = liquid chromatography) separation, the volumes of peptide containing solutions were adjusted to 7μl by addition of a 0.1 % (v/v) formic acid solution. Samples were settled in a Triathlon autosampler (Spack, Emmen, Holland). For each experiment, 5 μl of peptide containing solution were injected on a C18 reverse phase column of 75 μm inner diameter (YMS-ODS-AQ200, Michrom Bioresource, Auburn, CA). Peptides were eluted with an acetonitrile (ACN) gradient in the presence of 0.1 % (v/v) formic acid, using SunFlow pumps (SunChrom, Friderichsdorf, Germany). A flow splitter was used in order to decrease the flow rate after the pumps from 200 to 0.4 μl/min. Peptides were analysed with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Wythenshawe, England). A 2700 V tension was applied on the nanoelectrospray capillary (New Objective, Woburn, MA, USA). Argon was used as collision gas. The collision energy was settled as a function of the precursor ion mass. MS/MS spectra were acquired by automatic switching between MS and MS/MS mode. Acquired MS/MS data were

converted in a compatible format (DTA files) by ProteinLynx software (Micromass, Wythenshawe, England) and analysed using conventional search engines against SWISS-PROT, TrEMBL, NCBInr and EST databases. In cases of manual interpretation of MS/MS data, identification was performed by sequence only search.

It was found that marker M5 was a fragment of Cystatin C, markers M3 and M4 were isoforms of beta-2-microglobulin and M2 was a new variant or homologue of myoglobin. Marker M1 was found to be a fragment of the neurosecretory protein VGF.

Example 9: Statistical Analysis

P-values were calculated using standard statistical methods known to the person skilled in the art. P-values smaller than 0.05 were considered to be statistically significant.

Example 10: Isolation of the 4.8 kDa Fragment (Marker M1)

Some CSF samples from control patients were fractionated by Centricon 30 filtration device (Millipore Corp., Bedford, MA) in order to remove the protein with a molecular weight higher than 30 kDa. The salt and polypeptide with a molecular weight lower than 3 kDa were removed using a Centricon 3 (Millipore Corp., Bedford, MA). The Centricon 3 was then washed with ultrapure distilled water. In that wash fraction, the 4.82 kDa was found to be the major component. This liquid fraction was first reduced with a 10 mM solution of 1,4-Dithioerythritol for 1h at 56°C, then alkylated with 54 mM iodoacetamide for 45 min at room temperature. Finally, the polypeptide was digested with 6 mg/l trypsin overnight at 37°C. This liquid fraction was analysed by nanoLC and Q-TOF as previously described.

- 23 -

Example 11: Different Surface Materials for SELDI-TOF Analysis

Using SELDI-TOF, an analysis of 10 CSF samples from AD patients and 10 controls was performed on three different surfaces: the hydrophobic H50, the WCX2, and the

5   IMAC surface (Ciphergen Biosystems, Fremont, California, USA, resp.). Seven differentially expressed peaks were found on the H50, five markers on the WCX2, and five markers on the IMAC surface. A diagnostic test using the markers on the H50 chip revealed a specificity and sensitivity of 100% and 70%, respectively. The combination of the markers found on H50 and WCX2 gave a specificity and

10   sensitivity of 100% and 80%. Finally, the combination of the markers found on H50, WCX2 and IMAC gave a specificity and sensitivity of 100% and 90%.

The average masses of the differentially expressed polypeptides as determined by SELDI-TOF using different surface materials were as follows:

15

Surface hydrophobic H50: 7 peaks
Marker 1: 4769 ± s.d. Da
Marker 2: 6958 ± s.d. Da
Marker 3: 6991 ± s.d. Da
20   Marker 4: 13412 ± s.d. Da
Marker 5: 13787 ± s.d. Da
Marker 6: 17276 ± s.d. Da
Marker 7: 40437 ± s.d. Da

25   Surface IMAC Cu: 5 peaks
Marker 1: 6895 ± s.d. Da
Marker 2: 6928 ± s.d.Da
Marker 3: 7691 ± s.d. Da
Marker 4: 7769 ± s.d. Da
30   Marker 5: 7934 ± s.d. Da

- 24 -

Surface WCX2: 5 peaks

Marker 1: 5082 ± s.d. Da

Marker 2: 6267 ± s.d. Da

Marker 3: 6518 ± s.d. Da

5   Marker 4: 7274 ± s.d. Da

Marker 5: 8209 ± s.d. Da

The standard deviation (s.d.) is 20 Da for each marker above. However, the standard deviation can also be 40 Da, or 10 Da, or 5 Da for each marker above.

10

Bibliography

Adam, B.L. et al. 2001, Proteomics 1(10): 1264-1270

15   Asgeirsson B, Haebel S, Thorsteinsson L, Helgason E, Gudmundsson KO, Gudmundsson G, Roepstorff P. Hereditary cystatin C amyloid angiopathy: monitoring the presence of the Leu68Gln cystatin C variant in cerebrospinal fluid and monocyte cultures by MS. Biochem J. 1998, 329, 497-503.

20   Beyer K, Lao JI, Gomez M, Riutort N, Latorre P, Mate JL, Ariza A. Alzheimer's disease and the cystatin C gene polymorphism: an association study. Neuroscience letters. 2001, 315, 17-20.

Canu N, Possenti R, Ricco AS, Rocchi M, Levi A. Cloning, structural organization

25   analysis, and chromosomal assignment of the human gene for the neurosecretory protein VGF. Genomics 1997, 45 (2), 443-446.

Cohen DH, Feiner H, Jensson O, Frangione B. Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis (HCHWA-I) is related to the gastroentero-pancreatic

30   neuroendocrine protein. *J. Exp. Med.* 1983, 158, 623-628.

- 25 -

Current Protocols in Immunology, Eds. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W., John Wiley & Sons Inc. 1991

5   Crawford U, Freeman MJ, Schinka JA, Abdullah LI, Gold M, Hartman R, Krivian K, Morris MD, Richards D, Duara R, Anand R, Mullan MJ. A polymorphism in the cystatin C gene is a novel rsik factor for late-onset Alzheimer's disease. *Neurology*. 2000, 55, 763-768.

10   Davies, H.A. 2000. The ProteinChip(R) System from Ciphergen: A new technique for rapid, micro-scale protein biology. *J. Molecular Medicine*, 78 (7):B29 Deng A, Irizarry MC, Nitsch RM, Growdon JH, Rebeck GW. Elevation of Cystatin C in susceptible neurons in Alzheimer's disease. *Am. J. Pathol.* 2001, 159, 1061-1068.

15   Emerudh J, Olsson T, Berlin G, von Schenck H. Cerebrospinal fluid immunoglobulins and beta 2-microglobulin in lymphoproliferative and other neoplastic diseases of the central nervous system. *Arch Neurol.* 1987, 44(9), 915-20.

       Finckh U, Von der Kammer H, Velden J, Michel T, Andresen B, Deng A, Zhang J, Muller-Thomsen T, Zuchowski K, Menzer G, Mann U, Papassotiropoulos A, Heun
20   R, Zurdel J, Holst F, benussi L, Stoppe G, Reiss J, Miserez AR, Staehelin HB, Rebeck W, Hyman BT, Binetti G, Hock C, Growdon JH, Nitsch RM. Genetic association of a cystatin C gene polymorphism with late-onset Alzheimer's disease. *Arch Neurol.* 2000, 57, 1579-1583.

25   Francis, P.T. et al. J. Neurol. Neurosurg. Psychiatry 1999, 66(2): 137-147
       Fung, ET et al. Protein biochips for differential profiling. *Current Opinion in Biotechnology*, 2001, 12(1): 65-69

       Ghiso J, Jensson O, Frangione B. Amyloid fibrils in hereditary cerebral hemorrhage
30   with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C). *Proc Natl Acad Sci U S A.* 1986;83(9):2974-8.

- 26 -

Ghiso J, Pons-Estel B, Frangione B. Hereditary cerebral amyloid angiopathy: the amyloid fibrils contain a protein which is a variant of cystatin C, an inhibitor of lysosomal cysteine proteases. Biochem Biophys Res Commun. 1986;136(2):548-54.

Gras R, Müller M, Gasteiger E, Gay S, Binz PA, Bienvenut WV, Hoogland C, Sanchez JC, Bairoch A, Hochstrasser DF, Appel R. Improving protein identification from peptide mass fingerprinting through a parameterised multi-level scoring algorithm and an optimised peak detection. *Electrophoresis* 1999, 20: 3535-3550

Grubb AO, Jensson O, Gudmundsson G, Arnason A, Lofberg H, Malm J. Abnormal metabolism of g-trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. *N. Engl. J. Med* 1984, 311, 1547-1549.

Hellman U, Wernstedt C, Góñez, J, Heldin CH. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* 1995, 224, 451-5.

Hoekman K, Van Nieuwkoop JA, Willemze R. The significance of beta-2 microglobulin in clinical medicine. *Neth J Med.* 1985;28(12):551-7.

Huse WD, Sastry L, Iverson SA, Kang AS, Alting-Mees M, Burton DR, Benkovic SJ, Lerner RA. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. 1989, *Science* 246: 1275-1281.

Issaq HJ, Veenstra TD, Conrads TP, Felschow D. The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochem. Biophys. Res. Commun.* 2002, 292 (3): 587-592

Kalman J, Marki-Zay J, Juhasz A, Santha A, Dux L, Janka Z. Serum and Cerebrospinal fluid cystatin C levels in vascular and Alzheimer's dementia. *Acta Neurol. Scand.* 2000, 101, 279-282.

5   Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970 Aug 15;227(259):680-5.

Leung-Tack J, Tavera C, Gensac MC, Martinez J, Colle A. Modulation of phagocytosis-associated respiratory burst by human cystatin C: role of the N-terminal
10   tetrapeptide Lys-Pro-Pro-Arg. *Exp. Cell Research.* 1990, 188, 16-22.

Levy E, Sastre M, Kumar A, Gallo G, Piccardo P, Ghetti B, Tagliavini F, Codeposition of cystatin C with amyloid-beta protein in the brain of Alzheimer's disease patients. *J. Neuropathol Exp Neurol* 2001, 60, 94-104
15

Merchant M, Weinberger SR. . Recent advancements in surface-enhanced laser   . desorption/ionization-time of flight-mass spectrometry. *Electrophoresis*, 2000, 21: 1164-1167

20   Popovic T, Brzin J, Ritonja A, Turk V. Different forms of human Cystatin C. *Biol. Chem. Hoppe-Seyler.* 1990, 371, 575-580.

Raymackers J, Daniels A, DeBrabandere V, Missiaen C, Dauwe M, Verhaert P, Vanmechelen E, Meheus L. Identification of two dimensionally separated human
25   cerebrospinal fluid proteins by N-terminal sequencing, matrix-assisted laser-desorption/ionization-mass spectrometry, nanoliquid chromatography-electrospray ionization-time of flight-mass spectrometry, and tandem mass spectrometry. *Electrophoresis*, 2000, 21, 2266-2283.

30   The Ronald and Nancy Reagan Research Institue of the Alzheimer's Association and the National Institute on Aging Working Group. Consensus Report of the

- 28 -

Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease". *Neurobiology of Aging*, 1988, 19 (2): 109–116.

Robles A. . Some Remarks on biological markers of Alzheimer's Disease. *Neurobiology of Aging*, 1998, 19 (2): 153-157

Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal. Biochem.* 1992, 203, 173-9.

Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 1996, 68, 850-8.

Teunissen CE, de Vente J, Steinbusch HWM, De Bruijn C.. *Neurobiology of Aging*, 2002, 23: 485-508

US Patent 4,366,241
US Patent 4,376,110
US Patent 4,517,288
US Patent 4,837,168

Ward ES et al., Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. *Nature* 1989, 341, 544-546

Wei L, Berman Y, Castano EM, Cadene M, Beavis RC, Devi L, Levy E. Instability of the amyloidogenic cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type. *J. Biol. Chem.* 1998, 273, 11806-11814.

Wulfkuhle JD, McLean KC, Paweletz CP, Sgroi DC, Trock BJ, Steeg PS, Petricoin EF . New approaches to proteomic analysis of breast cancer. *Proteomics* 2001, 1 (10): 1205-1215

Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF, Dalmasso EA, Wright GL. Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. .2001, *Cancer Research* 61 (16): 6029-6033